Journal of Chromatography, 343 (1985) 119—127
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2668

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATION OF A *meta*-O-DEALKYLATED METABOLITE OF FLECAINIDE ACETATE, A NEW ANTIARRHYTHMIC

S.F. CHANG*, T.M. WELSCHER, A.M. MILLER, R.L. McQUINN and J.M. FOX

Riker Laboratories, Inc., Subsidiary of 3M, St. Paul, MN 55144 (U.S.A.)

(First received November 7th, 1984; revised manuscript received April 16th, 1985)

SUMMARY

A simple, sensitive and selective high-performance liquid chromatographic method has been developed to analyze *meta*-O-dealkylated flecainide, a major metabolite of flecainide acetate, in human biological fluids. Sample preparation is accomplished through the use of bonded-phase extraction columns and the samples are chromatographed on a reversed-phase system with fluorescence detection. An external calibration method is used for quantitation and the inter-day and intra-day precision and accuracy are good. The method has been used to determine metabolite levels in samples from healthy subjects and patients with arrhythmias or renal impairment.

INTRODUCTION

Flecainide acetate (Tambocor®, R-818) is a new antiarrhythmic that has been shown to be effective for the suppression of ventricular arrhythmias [1—4]. Flecainide acetate is currently undergoing extensive clinical evaluation world-wide and is marketed in several countries overseas including Germany and the U.K. Two major metabolites of flecainide have been isolated from human urine and identified. One of the major metabolites is *meta*-O-dealkylated flecainide (MODF, 5-hydroxy-N-(2-piperidylmethyl)-2-(2,2,2-trifluoroethoxy)-benzamide, Fig. 1). Since MODF also exhibits some weak antiarrhythmic activity in laboratory animal models [5], a sensitive and selective method was developed to assess metabolite levels in human biological fluids.

This paper describes a high-performance liquid chromatographic (HPLC) method for the quantitation of MODF in human plasma, and in enzyme treated and untreated urine and dialysate. Sample preparation is accomplished

0378-4347/85/$03.30

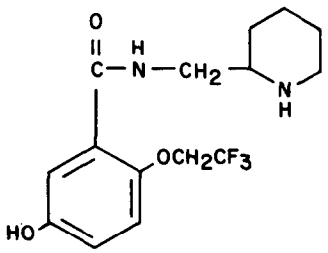

Fig. 1. Structure of 5-hydroxy-N-(2-piperidylmethyl)-2-(2,2,2-trifluoroethoxy)benzamide (MODF).

through the use of bonded-phase extraction columns, and the separation and quantitation are performed with a reversed-phase column and fluorescence detector. This procedure is sensitive and selective, and requires a minimum of operator time and handling.

EXPERIMENTAL

Chemicals and reagents

MODF was synthesized in-house [6]. The phosphoric acid and sodium carbonate were analytical reagent grade. The acetonitrile and methanol were Omnisolv® (EM Science, Gibbtown, NJ, U.S.A.). The water was deionized.

Preparation of standard solutions

Standard solutions were prepared by diluting a 10 mg MODF per 10 ml of 0.01 *M* hydrochloric acid stock solution. The concentrations used were 10, 15, 20, 30, 50, 75, 100, 200, 300 and 800 ng MODF per 50 μl of 0.01 *M* hydrochloric acid. The solutions were stored at room temperature with no sign of degradation for up to six months.

Apparatus

The liquid chromatographic system consisted of a Constametric III pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a WISP 710B automatic sample injector (Waters Assoc., Milford, MA, U.S.A.), and a Schoeffel 970 fluorescence detector (Schoeffel Instrument, Westwood, NJ, U.S.A.). The excitation and emission wavelengths were 230 nm and 340 nm, respectively. Data were collected by a strip chart recorder (Linear Instruments, Irvine, CA, U.S.A.).

The mobile phase was prepared by mixing acetonitrile and 0.06% aqueous phosphoric acid in varying percentages and filtering through a 0.45-μm Nylon 66 filter (Alltech Associates, Deerfield, IL, U.S.A.). Three elution systems were used for chromatographing the different sample types. System I: Waters phenyl column, 30 cm × 4.6 mm, 10 μm particle size (Waters Assoc.) with a mobile phase of acetonitrile—0.06% aqueous phosphoric acid (24:76, v/v). This system was used for plasma and untreated dialysate. System II: Waters phenyl column with a mobile phase ratio of 21:79. This system was used for untreated urine. System III: Supelco LC-8 column, 15 cm × 4.6 mm, 5 μm particle size (Supelco, Bellefonte, PA, U.S.A.) with a mobile phase ratio of 36:64. This system was used for enzyme treated urine and dialysate. The flow-rate for all systems was 2 ml/min, and all analyses were performed at room temperature.

The vacuum manifold used was from Analytichem International (Harbor City, CA, U.S.A.) and the disposable extraction columns were filled with reversed-phase octyl packing, 200 mg (J.T. Baker, Phillipsburg, NJ, U.S.A.).

Enzyme treatment

Aliquots of urine and dialysate samples were incubated with β-glucuronidase/aryl sulfatase (Calbiochem, La Jolla, CA, U.S.A.), to hydrolyze the conjugated metabolite so that total levels of MODF could be determined. The procedure used was: add to 2.0 ml of urine or dialysate, 10 μl of 20% acetic acid, 100 μl of 2 *M* sodium acetate buffer (pH 5.3), 40 μl of β-glucuronidase/aryl sulfatase (0.34 IU/0.07 IU) and 8 μl of chloroform; the mixture was then incubated with gentle shaking at 37°C for about 24 h [5]. The hydrolysis of MODF was essentially complete using this procedure.

Extraction

Each extraction column is initially activated by wetting with 2 column vols. of methanol and then with 2 column vols. of water. Once activated, care must be taken to avoid drying the column before the sample has been loaded.

Plasma and dialysate (treated and untreated). Pipet onto the top of the extraction column: 1 ml plasma, 3 ml dialysate, or 0.5 ml enzyme-treated dialysate. This is followed by 50 μl of 0.01 *M* hydrochloric acid (replaced with standard solution for the calibration curve) and 0.2 ml of 0.2 *M* sodium carbonate. The mixture is stirred with the closed end of a capillary tube and drawn through the column by a water aspirator. Each column is then washed with 2 column vols. of water and three aliquots of 1 ml acetonitrile. Collection tubes (2 ml conical tubes) are placed in the vacuum manifold and the MODF is eluted with 500 μl of methanol. The methanolic eluent is evaporated to dryness under a stream of nitrogen at 40°C. The residue is reconstituted in 50 μl of mobile phase, and 25 μl are injected into the HPLC system.

Urine (treated and untreated). The urine and enzyme treated urine samples require a dual-column extraction procedure to adequately clean up the sample before chromatographic analysis. The procedure for the first column is the same as that described above except that a 0.5-ml sample is used and the residue in the final step is reconstituted in 200 μl of 0.01 *M* hydrochloric acid. This solution is then processed through a second column as described below.

A 1-ml aliquot of water is pipetted onto the top of the extraction column, followed by the aqueous mixture from the first column and 0.2 ml of 0.2 *M* sodium carbonate. The acetonitrile and water washes, elution of MODF and eluent evaporation is the same as that described in the procedure for plasma. The residue is reconstituted in 100 μl of mobile phase, and 25 μl are injected into the HPLC system.

Calibration and quantitation

A least-squares linear regression line of the peak height for MODF versus concentration was calculated. The slope and intercept of this line were used to calculate the MODF concentration in unknown samples. A set of calibration standards was run with each set of unknowns.

RESULTS AND DISCUSSION

Sample preparation

Use of the vacuum manifold allowed up to ten samples to be processed at any one time and each batch of samples required only 20 min to complete. Thus, the use of bonded-phase extraction columns is time efficient. Although the single column procedure sufficiently cleaned up the plasma and dialysate samples, it did not adequately clean up the urine samples. By processing the urine samples through a second extraction column, the interfering constituents were removed and quantitation was possible. The dual-column procedure did not reduce the recovery of MODF greatly, and the lower limit of quantitation remained the same. For the enzyme treated dialysate samples, a smaller sample volume (0.5 ml compared to 3 ml used for untreated dialysate samples) had to be used. The decreased sensitivity due to the smaller sample size was still adequate for the monitoring of total MODF in dialysate. Several methods are available for the analysis of the parent compound, flecainide [7—13], one method uses the same sample preparation procedure [7]. Thus theoretically, the parent compound and MODF could be analyzed simultaneously. However, in the elution systems for MODF, the parent compound had a very long retention time and it was impractical to routinely analyze these two compounds together. It might be possible to develop a gradient elution system to accomplish this, but the possibility has not yet been explored.

During the method development period, extensive effort was made to develop an internal standard procedure. The best internal standard candidate examined was the positional isomer of MODF, 2-hydroxy-N-(2-piperidylmethyl)-5-(2,2,2-trifluoroethoxy)benzamide. Since this compound might also be a metabolite of flecainide, it was specifically looked for and was not found in detectable concentrations [5]. Numerous experiments were carried out with this candidate internal standard and the results indicated that in most cases, calibration by the external standard method exhibited less variability than by the internal standard method.

Elution systems

Although the single or dual extraction procedures extensively cleaned up the biological samples, there was still a small number of interfering substances remaining. These interfering substances could be chromatographically separated; however, they appeared to vary among the different type of samples (plasma, dialysate or urine) and could not be separated from MODF by using one identical elution system. Therefore, for each type of sample, one of the three elution systems previously described was used. Sample chromatograms for plasma, urine and enzyme treated urine are shown in Figs. 2, 3 and 4, respectively.

Linearity and sensitivity

The concentration range of MODF in each type of sample varied, which necessitated the use of different concentration ranges for the calibration curves. The concentration range of the calibration curve for plasma was 10—50 ng/ml MODF with a 1-ml sample, and 3.3—16.7 ng/ml MODF with a 3-ml sample for

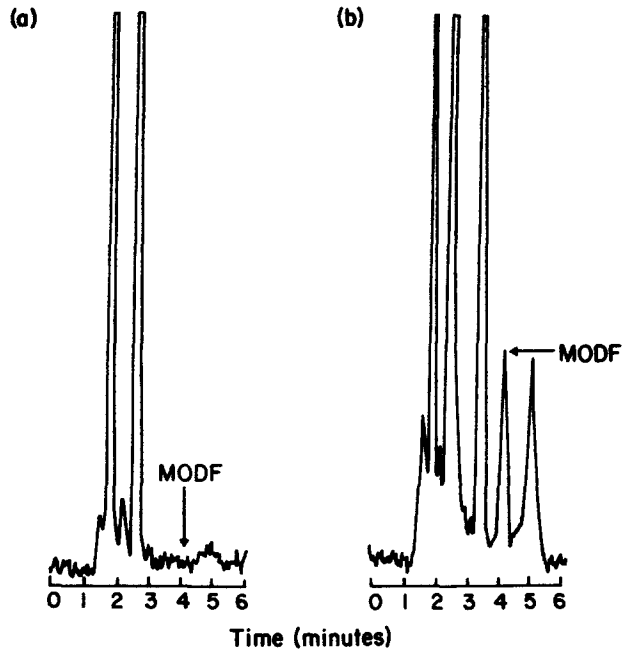

Fig. 2. Typical chromatograms of human plasma samples: (a) blank plasma; (b) plasma of patient dosed with flecainide acetate, concentration level is 41 ng/ml MODF.

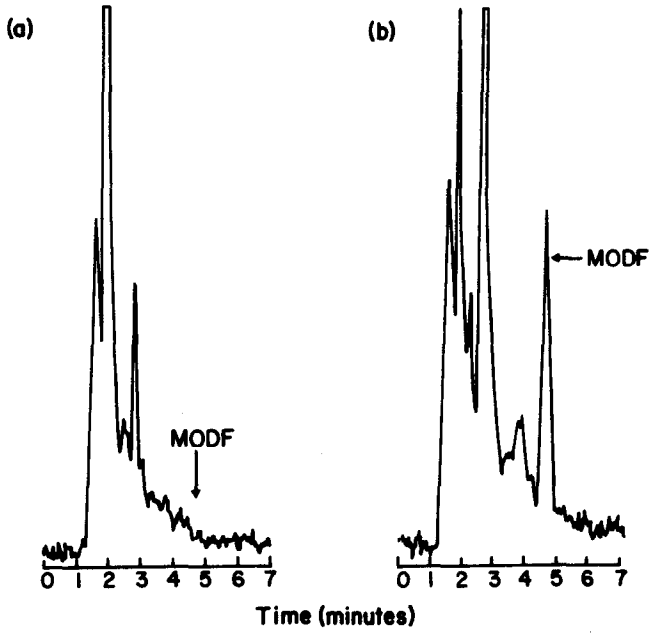

Fig. 3. Typical chromatograms of human urine samples: (a) blank urine; (b) urine of patient dosed with flecainide acetate, concentration level is 380 ng/ml MODF.

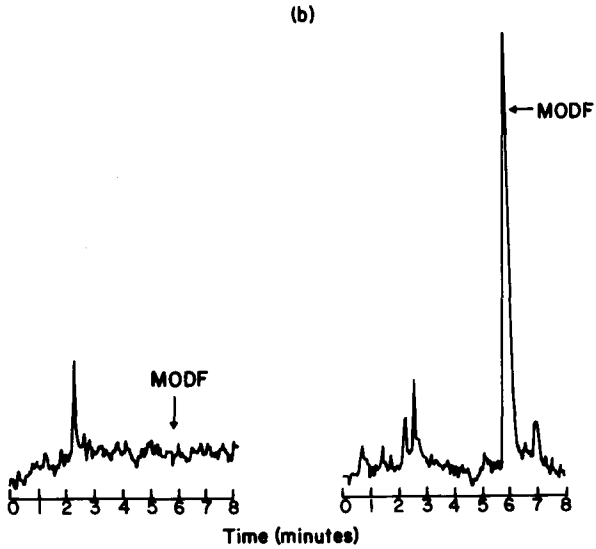

Fig. 4. Typical chromatograms of enzyme treated urine: (a) blank enzyme treated urine; (b) enzyme treated urine of patient dosed with flecanide acetate, concentration level is 3755 ng/ml MODF.

untreated dialysate. Since the urinary concentration range of MODF was higher than plasma and dialysate, a 100—800 ng/ml MODF concentration range (with a 0.5-ml sample volume) was used for both enzyme treated and untreated urine. For enzyme treated dialysate, a calibration curve of 20—100 ng/ml MODF with a 0.5-ml sample volume was established. The need for a smaller sample size resulting in a higher concentration range was necessitated by the presence of an interference peak. The lowest quantifiable concentration was the lowest calibration standard used in all sample types. The linear regression results for all the calibration curves were good with a correlation coefficient (r) value greater than 0.997 in all cases.

Precision and accuracy

The intra-day precision and accuracy for plasma were determined by analyzing replicate samples containing 10, 20, 50, 75 and 100 ng/ml MODF. The precision, expressed as coefficient of variation, was 9.9%, 4.3%, 3.1%, 2.7% and 4.7% for the concentrations 10, 20, 50, 75 and 100 ng/ml MODF, respectively. The accuracy, expressed as relative error, was 4.4%, 4.0%, 0.4%, 6.3% and 4.7% over the same concentration range. Table I contains the detailed results for intra-day precision and accuracy. The inter-day precision was determined by analyzing replicate samples over a period of ten weeks. The coefficient of variation range was 1.5—10.8% over the concentration range of 10—50 ng/ml MODF (Table II).

Inter-day precision and accuracy were also determined for dialysate. The range for the coefficient of variation was 5.9—10.7% over a concentration range of 3.3—16.7 ng/ml MODF. The range for the relative error was —9.6% to +6.9% over the concentration range mentioned above. The detailed results for dialysate are presented in Table III.

TABLE I

INTRA-DAY PRECISION AND ACCURACY FOR PLASMA (n = 5)

Concentration (ng/ml)	Mean ± S.D. (ng/ml)	Coefficient of variation (%)	Relative error (%)
10	10.4 ± 1.0	9.9	4.4
20	20.8 ± 0.9	4.3	4.0
50	50.2 ± 1.6	3.1	0.4
75	79.7 ± 2.2	2.7	6.3
100	104.7 ± 4.9	4.7	4.7

TABLE II

INTER-DAY PRECISION FOR PLASMA

Concentration (ng/ml)	Number of samples	Mean ± S.D. (ng/ml)	Coefficient of variation (%)
10	11	10.7 ± 1.2	10.8
15	11	15.3 ± 1.7	10.8
20	10	19.7 ± 1.0	4.9
30	11	30.2 ± 1.4	4.7
50	11	49.8 ± 0.8	1.5

TABLE III

INTRA-DAY PRECISION AND ACCURACY FOR DIALYSATE

n = 5.

Concentration (ng/ml)	Mean ± S.D. (ng/ml)	Coefficient of variation (%)	Relative error (%)
3.3	3.4 ± 0.2	5.9	0.6
5.0	4.9 ± 0.4	8.1	−2.5
6.7	7.1 ± 0.5	6.8	6.9
10.0	10.2 ± 1.1	10.7	1.9
16.7	15.1 ± 1.0	6.8	−9.6

Application

This method has been successfully used for quantitation in plasma and urine of healthy subjects and in plasma, urine and dialysate of patients with arrhythmias or renal impairment. In healthy human volunteers, plasma levels of MODF after a single, 200-mg oral dose of flecainide were generally very low (< 25 ng/ml), and MODF was rapidly cleared from plasma. In patients on a multiple oral dose regimen with flecainide acetate (100 mg twice daily to 300 mg twice daily), trough plasma levels of MODF were found to be very low, relative to measured flecainide levels, and plasma MODF levels did not appear to accumulate with repeated dosing (Table IV). MODF has previously been shown to be extensively excreted in human urine as a conjugate that, in the presence

TABLE IV

TROUGH PLASMA CONCENTRATIONS OF FLECAINIDE AND MODF FOLLOWING MULTIPLE ORAL DOSAGE TO OUTPATIENTS

Patient No.	Flecainide dosage regimen (mg b.i.d.)	Outpatient day 7		Outpatient day 14	
		Flecainide levels (ng/ml)	MODF levels (ng/ml)	Flecainide levels (ng/ml)	MODF levels (ng/ml)
1	100	459	**	439	**
2	200	1194	13, 11***	1198	15, 16***
3	200	886	32	1086	41
4	200	390	*	374	*
5	300	755	28	630	28
6	250	352	*	345	*
7	250	1020	15	1039	12
8	250	*	*	859	30
9	200	1137	28	1226	29
10	100 (t.i.d.)	750	16	907	18
11	200	292	13	269	13

*Sample not available.
**Below standard curve.
***Random repeat.

TABLE V

URINARY EXCRETION OF FREE (NOT CONJUGATED) AND TOTAL (FREE PLUS CONJUGATED) MODF IN HUMANS FOLLOWING ORAL ADMINISTRATION OF A SINGLE, 200-mg DOSE OF FLECAINIDE

Urine collection interval postdose (h)	Free (not conjugated MODF)			Total (free plus conjugated) MODF		
	Concentration in urine* (ng/ml)	Total amount in urine** (mg)	Percent of flecainide dose in urine*** (%)	Concentration in urine§ (ng/ml)	Total amount in urine** (mg)	Percent of flecainide dose in urine*** (%)
0—24	3376	4.82	3.44	13021	18.62	13.29
24—48	842	1.10	0.78	3818	4.96	3.54
48—72	238	0.36	0.26	795	1.20	0.86
72—96	75	0.11	0.08	429	0.62	0.44
Cumulative (0—96)		6.39	4.56		25.40	18.13

*Mean value for duplicate determinations; no MODF was detected in the predose urine pool.
**Based on the mean 24-h urine volume for the sixteen subjects, the average amount per subject.
***Corrected for the difference in molecular weight between the MODF (332.2) and flecainide acetate (474.4).
§Single determination; no MODF was detected in the predose urine pool.

of the enzyme β-glucuronidase/aryl sulfatase, is completely hydrolyzed to the aglycone [5]. After a single, 200-mg oral dose of flecainide acetate to sixteen healthy volunteers, the cumulative excretion of MODF was measured with and without enzyme pretreatment and was found to be on the average 18.1% and 4.6% of the dose, respectively (Table V).

CONCLUSION

An HPLC method has been developed that can monitor MODF at low nanogram levels in human plasma, and enzyme treated and untreated urine and dialysate. The method is sensitive, selective and simple; twenty samples can be processed and loaded onto the liquid chromatograph in approximately 1 h. Although the method uses external calibration, the intra-day and inter-day precision and accuracy results are good. The method has been used to monitor MODF levels in ongoing clinical studies.

ACKNOWLEDGEMENTS

The authors wish to acknowledge G.J. Quarfoth for providing the β-glucuronidase/aryl sulfatase treated urine and dialysate, G.J. Conard for critically reviewing the manuscript, and J.E. Warn and C.J. Belisle for typing the manuscript.

REFERENCES

1 J.L. Anderson, J.R. Stewart, B.A. Perry, D.D. van Hamersveld, T.A. Johnson, G.J. Conard, S.F. Chang, D.C. Kvam and B. Pitt, N. Engl. J. Med., 305 (1981) 473.
2 H.J. Duff, D.M. Roden, R.J. Maffucci, B.S. Vesper, G.J. Conard, S.B. Higgins, J.A. Oates, R.F. Smith and R.L. Woosley, Amer. J. Cardiol., 48 (1981) 1133.
3 M. Hodges, J.M. Haugland, G. Granrud, G.J. Conard, R.W. Asinger, F.L. Mikell and J. Krejci, Circulation, 65 (1982) 879.
4 Flecainide-Quinidine Research Group, Circulation, 67 (1983) 1117.
5 R.L. McQuinn, G.J. Quarfoth, J.D. Johnson, E.H. Banitt, S.V. Pathre, S.F. Chang, R.E. Ober and G.J. Conard, Drug Metab. Dispos., 12 (1984) 414.
6 E.H. Banitt, W.R. Bronn, M.T. Case, G.J. Conard and J.R. Schmid, Flecainide Acetate; A New Fluorine Containing Antiarrhythmic Agent, presented at the 188th National Meeting of the American Chemical Society, Aug. 26, 1984, Philadelphia, PA, U.S.A.
7 S.F. Chang, A.M. Miller, J.M. Fox and T.M. Welscher, Ther. Drug Monitor., 6 (1984) 105.
8 J.D. Johnson, G.L. Carlson, J.M. Fox, A.M. Miller, S.F. Chang and G.J. Conard, J. Pharm. Sci., 73 (1984) 1469.
9 S.F. Chang, A.M. Miller, M.J. Jernberg, R.E. Ober and G.J. Conard, Arzneim-Forsch, 33 (1983) 251.
10 K.A. Muhiddin and A. Johnston, Brit. J. Clin. Pharmacol., 12 (1981) 283.
11 J.W. De Jong, J.A.J. Hegge, E. Harmsen and P.Ph. De Tombe, J. Chromatogr., 229 (1982) 498.
12 S.F. Chang, T.M. Welscher, A.M. Miller and R.E. Ober, J. Chromatogr., 272 (1983) 341.
13 S.F. Chang, A.M. Miller, J.M. Fox and T.M. Welscher, J. Liquid Chromatogr., 7 (1984) 167.